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A new species of *Gracixalus* (Amphibia, Anura, Rhacophoridae) from Guizhou Province, China

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Abstract

A new species of small tree frog was discovered in southwestern China, exhibiting morphological differences and molecular divergence. The new species, formally described herein as *Gracixalus weii* **sp. nov.**, is morphologically distinct from other congeners by body size (SVL 30.1–34.0 mm in males and 35.2–36.0 mm in females). In addition, the head width was larger than the head length, with a distinct tympanum, a brown to beige dorsum, and an inversed Y-shaped dark brown marking. The skin of the dorsal and lateral surfaces of the head, body, and limbs was rough and sparsely scattered with tubercles. The toes showed moderately developed webbing, while finger webbing was rudimentary. Nuptial pads were found on the base of finger I; males had a single subgular vocal sac, with heels overlapping when the legs were at a right angle to the body, and the tibiotarsal articulation reached the mid-eye when the legs were stretched forward. Phylogenetic analyses based on DNA sequences of the mitochondrial 16S rRNA supported the new species as a sister species of *G. jinxiuensis*. Genetically, the new species diverges from its congeners by 3.4%–14.5% (uncorrected p-distance) and is closest to *G. jinxiuensis*, which is 3.4%.

Key Words

Gracixalus weii sp. nov., Rhacophoridae, taxonomy, mitochondrial gene, southwestern China

Introduction

The genus *Gracixalus* Delorme, Dubois, Grosjean & Ohler, 2005, was characterized as a distinct genus by the molecular phylogenetic research of Li et al. (2008). Taxonomic assignments of the group have been highly controversial for a long time (Delorme et al. 2005; Li et al. 2008; Chen et al. 2020; Dubois et al. 2021). Delorme et al. (2005) established the new subgenus *Gracixalus* with *Philautus gracilipes* Bourret, 1937, as the type species, and placed it under *Aquixalus* Delorme, Dubois, Grosjean, & Ohler, 2005. Li et al. (2008), based on a larger molecular dataset, suggested that *Gracixalus* should be elevated to the genus level, a view supported by related studies (Pyron and Wiens 2011; Li et al. 2013). However, Chen et al. (2020) argued that the position of *Gracixalus* is uncertain. Subsequently, Dubois et al. (2021) partitioned *Gracixalus* into *Orixalus* Dubois, Ohler, & Pyron, 2021, and *Gracixalus* as sister genera. This view has also been recognized by the website of Amphibian Species of the World (Frost 2024), which categorizes the *Gracixalus* genus within the Rhacophorinae family into two genera: *Gracixalus* and *Orixalus*. The *Gracixalus* genus includes 16 species, while the *Orixalus* genus includes 4 species.

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But this division has not gained widespread recognition (Yu et al. 2019; Boruah et al. 2023; Tran et al. 2023), and recent research continues to support the classification of these species as a single genus, all belonging to the *Gracixalus* genus. The recent discovery of two new species, *G. patkaiensis* Boruah, Deepak, Patel, Jithin, Yomcha, & Das, 2023, and *G. truongi* Tran, Pham, Le, Nguyen, Ziegler, & Pham, 2023, further reinforces this unified classification (Boruah et al. 2023; Tran et al. 2023). Consequently, the research herein presents the *Gracixalus* genus as encompassing 20 species.

Geographically, the *Gracixalus* genus is dispersed from Myanmar and western Thailand to Laos, central and northern Vietnam, and further to southern and southwestern China (Boruah et al. 2023; Tran et al. 2023). These species of *Gracixalus i*nhabit elevations that span from 200 to 2500 m, and the majority of these species have been described in recent years (Frost 2024), indicating that the species diversity of *Gracixalus* has been poorly understood. Furthermore, recent phylogenetic studies conducted by Matsui et al. (2017) and Chen et al. (2018) have identified several unnamed, distinct lineages within the group of *G. jinxiuensis*. These findings suggest that the species richness of *Gracixalus* is still underestimated.

During recent fieldwork in Leigongshan Nature Reserve, Guizhou Province, China, specimens of a small-sized tree frog were collected. Upon reviewing relevant literature, it was found that there has been no previous record of the distribution of this species within the reserve, nor even within Guizhou Province. Morphological examination revealed that these specimens closely resembled *G. jinxiuensis* but exhibited notable differences. These included larger size, overlapping heels when the thighs were positioned perpendicular to the body, and the tibiotarsal articulation reaching the mid-eye level when the legs were extended forward. Comprehensive phylogenetic analyses were conducted using mitochondrial DNA and morphological comparisons to delineate these specimens further. These analyses consistently confirmed that the specimens from the Leigongshan Nature Reserve represent a previously undescribed taxon, which is hereby described as a new species.

Materials and methods

Sampling

Two adult females and five adult males of the undescribed species were collected from Leishan County, Guizhou Province, China (Fig. 1; Table 1). And the fieldwork was approved by the Management Office of the Leigongshan Nature Reserve (project number: P52260020244000011). The Animal Care and Use Committee of Guizhou University provided full approval for this research (Number: EAE-GZU-20244-T1228). The collected specimens were first anesthetized using an anesthetic, followed by specimen preparation, photography, and muscle sampling, ensuring that the harm to the animals was minimized and there were no ethical issues related to animal welfare. The frogs were firstly euthanized using isoflurane, and then the specimens were fixed in 75% ethanol for preservation. Tissue samples were taken and preserved separately in 95% ethanol prior to fixation. Specimens collected in this work were deposited in the Moutai Institute (**MT**), Guizhou Province, China.

Collection of molecular data

DNA was extracted from tissue using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). A fragment encoding partial 16S rRNA (16S) gene was amplified and sequenced. The primers used for 16S were P7 (5'-CGCCTGTTTACCAAAAACAT-3') and P8 (5'-CCGGTCTGAACTCAGATCACGT') following Simon et al. (1994). PCR amplification reactions were performed in a 30 µl reaction with the following cycling conditions: an initial denaturing step at 95 °C for 4 min; 35 cycles of denaturing at 95 °C for 40 s, annealing at 50 °C for 40 s, and extending at 72 °C for 70 s; and a final extending step of 72 °C for 10 min. PCR products were purified with spin columns and then were sequenced with both forward and reverse primers, the same as PCR. Sequencing was conducted using an ABI Prism 3730 automated DNA sequencer at Chengdu TSING KE Biological Technology Co. Ltd. (Chengdu, China). This study collected seven *Gracixalus* specimens according to the above procedures and were sequenced; all sequences were deposited in GenBank (for GenBank Accession Nos., refer to Table 1). For phylogenetic analyses, we downloaded corresponding sequences for all related species from GenBank according to previous studies (Yu et al. 2019; Boruah et al. 2023; Tran et al. 2023; for GenBank Accession Nos. PP852207–PP852213, refer to Table 1).

Phylogenetic analyses and genetic distance.

A total of 51 sequences (16S) were used for the molecular analyses, representing 24 species of subfamily Rhacophoridae. Contains seven sequences were derived from muscle tissue samples from the specimens collected in this study, and 44 sequences were downloaded from GenBank (Table 1). Following Matsui et al. (2017), *Rhacophorus borneensis* (Matsui, 2013) and *Kurixalus idiootocus* (Kuramoto & Wang, 1987) were used as outgroups in this study. All sequences were assembled and aligned using the Clustalw module in BioEdit 7.0.9.0 (Hall 1999) with default settings. Based on the 16S dataset, phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) methods, implemented in PhyML 3.0 (Guindon et al. 2010) and MrBayes 3.12 (Ronquist and Huelsenbeck 2003), respectively. The best-fit model was obtained by the Bayesian information criteria (BIC) computed with

Table 1. Information of samples used in molecular phylogenetic analyses in this study; a slash (/) indicates information absent.

ID	Species	Locality	Voucher number	GenBank accession number
1	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20230805003	PP852207
2	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240517001	PP852208
3	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240517002	PP852209
4	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240517003	PP852210
5	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240518008	PP852211
6	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240518009	PP852212
7	*Gracixalus weii* sp. nov.	Leigongshan, Leishan, Guizhou, China	MT LS20240518010	PP852213
8	*Gracixalus ananjevae*	Nghe An, Vietnam	VNMN 03012	JN862546
9	*Gracixalus carinensis*	Lao Cai, Vietnam	KUHE:46402	LC011939
10	*Gracixalus gracilipes*	Pingbian, Yunnan, China	060821196Rao	GQ285668
11	*Gracixalus gracilipes*	Ha Giang, Vietnam	AMNHA163897	DQ283051
12	*Gracixalus guangdongensis*	Mt.Nankun,Guangdong, China	SYS a004688	MG520191
13	*Gracixalus guangdongensis*	Mt.Nanling,Guangdong, China	SYS a004689	MG520192
14	*Gracixalus guangdongensis*	Mt.Nanling,Guangdong, China	SYS a004902	MG520193
15	*Gracixalus guangdongensis*	Dawuling,Guangdong, China	SYS a004904	MG520195
16	*Gracixalus guangdongensis*	Dawuling,Guangdong, China	SYS a005751	MG520196
17	*Gracixalus guangdongensis*	Dawuling,Guangdong, China	SYS a005750	MG520197
18	*Gracixalus guangdongensis*	Yizhang,Hunan, China	CIB HN201108200	LC011936
19	*Gracixalus jinxiuensis*	Jinxiu, Guangxi, China	SYS a002182	KY624584
20	*Gracixalus jinxiuensis*	Jinxiu, Guangxi, China	KIZ 060821013	EF564524
21	*Gracixalus jinxiuensis*	Jinxiu, Guangxi, China	KIZ 061210YP	EU215525
22	*Gracixalus jinxiuensis*	Jinxiu, Guangxi, China	SYS a002183	KY624585
23	*Gracixalus jinggangensis*	Mt. Jinggang, Jiangxi,China	SYS a003186	KY624587
24	*Gracixalus lumarius*	Kon Tum, Vietnam	AMS R 176202	KF918412
25	*Gracixalus medogensis*	Medog, Tibet, China	KIZ010956	MW023606
26	*Gracixalus nonggangensis*	Longzhou,Guangxi, China	SYSa005867	MG520202
27	*Gracixalus nonggangensis*	Longzhou,Guangxi, China	SYSa005868	MG520203
28	*Gracixalus nonggangensis*	Longzhou,Guangxi, China	SYSa005869	MG520204
29	*Gracixalus patkaiensis*	Changlang, Arunachal Pradesh, India	WII-ADA1352	OQ940026
30	*Gracixalus quangi*	Nghe An, Vietnam	AMS R173417	JN862539
31	*Gracixalus quyeti*	Cha Noi, Vietnam	VNUH 160706	EU871428
32	*Gracixalus sapaensis*	Lao Cai, Vietnam	MNHN 1999.5961	AY880503
33	*Gracixalus seesom*	Kanchanaburi, Thailand	KUHE 35084	LC011932
34	*Gracixalus* sp.	Wenshan, Yunnan, China	03320Rao	GQ285669
35	*Gracixalus supercornutus*	Gia Lai, Vietnam	AMS R176287	KT374016
36	*Gracixalus tianlinensis*	Tianlin,Guangxi, China	NHMG 1705015	MH117960
37	*Gracixalus tianlinensis*	Tianlin,Guangxi, China	NHMG 1705016	MH117961
38	*Gracixalus trieng*	Kon Tum Province, Vietnam	UNS 00342/AMS R176205	MT328245
39	*Gracixalus truongi*	Tuan Giao, Dien Bien, Vietnam	IEBR A.5004	OP750512
40	*Gracixalus yunnanensis*	Lvchuan, Yunnan, China	GXNU YU000060	MK234876
41	*Gracixalus yunnanensis*	Jinping, Yunnan, China	KIZ 060821126	EF564525
42	*Gracixalus yunnanensis*	Fazhanhe, Lancang, Yunnan, China	KIZ 20160228	MK234881
43	*Gracixalus yunnanensis*	Fudong, Lancang, Yunnan, China	KIZ 20160226	MK234880
44	*Gracixalus yunnanensis*	Xuelin, Lancang, Yunnan, China	KIZ 20160222	MK234878
45	*Gracixalus yunnanensis*	Bada, Menghai, Yunnan, China	KIZ 20160216	MK234877
46	*Gracixalus yunnanensis*	Lao Cai, Vietnam	VNMN 4355	LC140985
47	*Gracixalus yunnanensis*	Houapan, Laos	KUHE 32453	LC011937
48	*Gracixalus yunnanensis*	Nghe An, Vietnam	AMS R173454	JN862547
49	*Gracixalus ziegleri*	/	/	LC642812
50	*Kurixalus idiootocus*	Taiwan, China	KUHE 12979	AB933306
51	*Rhacophorus borneensis*	Sabah, Malaysia	BORN 22410	AB781693

PartitionFinder 2 (Lanfear et al. 2012). As a result, the GTR + G + I model was suggested as the best model for analysis. For ML analysis, the bootstrap consensus tree inferred from 1000 replicates was used to estimate nodal supports of inferred relationships on phylogenetic trees. For Bayesian analyses, we run four Markov chains for 50 million generations with sampling every 1000 generations, removing the first 25% of trees as the "burn-in" stage followed by calculation. The phylogenetic trees were visualized using FigTree 1.4.3 (Rambaut et al. 2014). Finally, we calculated the pairwise sequence divergence based on uncorrected *p*-distance using MEGA 6.06 (Tamura et al. 2013) to determine the genetic distance between the species of *Gracixalus* by 16S.

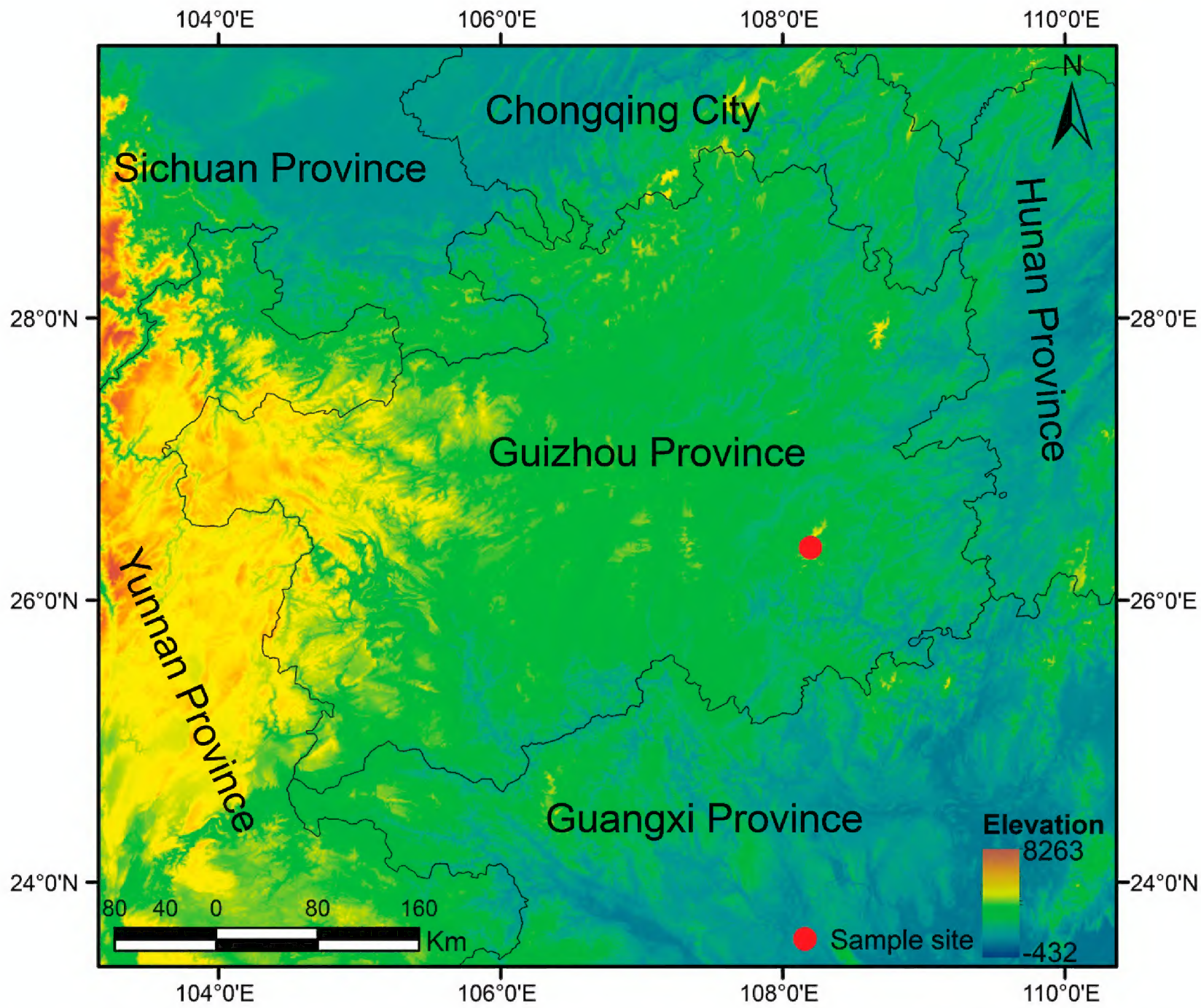

Figure 1. Geographical location of the type locality of *Gracixalus weii* sp. nov., Leishan County, Guizhou Province, China. The map was generated using ArcGIS 10.4 software.

Morphological comparisons

Morphological measurements were made with dial calipers to the nearest 0.1 mm by one of us (Shize Li) following Fei et al. (2009). A total of 18 morphological characteristics were measured, containing seven specimens of the undescribed taxon and two *G. jinxiuensis* (CIB58795/660386 and CIB110925) from the Chengdu Institute of Biology, Chinese Academy of Science, abbreviated as follows:

ED eye diameter (distance from the anterior corner to the posterior corner of the eye);
FL foot length (distance from tarsus to the tip of fourth toe);
HDL head length (distance from the tip of the snout to the articulation of the jaw);
HDW maximum head width (greatest width between the left and right articulations of the jaw);
HLL hindlimb length (maximum length from the vent to the distal tip of the Toe IV);
IND internasal distance (minimum distance between the inner margins of the external nares);
IOD interorbital distance (minimum distance between the inner edges of the upper eyelids);
LAL length of lower arm and hand (distance from the elbow to the distal end of the Finger IV);
ML manus length (distance from tip of third digit to the proximal edge of the inner palmar tubercle);
LW lower arm width (maximum width of the lower arm);
SVL snout-vent length (distance from the tip of the snout to the posterior edge of the vent);
SL snout length (distance from the tip of the snout to the anterior corner of the eye);
TFL length of foot and tarsus (distance from the tibiotarsal articulation to the distal end of the Toe IV);
THL thigh length (distance from the vent to the knee),
TL tibia length (distance from the knee to the tarsus);
TW maximal tibia width;
TYD maximal tympanum diameter;
UEW upper eyelid width (greatest width of the upper eyelid margins measured perpendicular to the anterior-posterior axis).

To reduce allometric effects, all measurements were size-corrected with respect to SVL prior to morphometric analysis. Principal component analyses (PCAs) of size-corrected measurements and simple bivariate scatterplots were used to explore and characterize the mor-

phometric differences between the new species and *G. jinxiuensis*. Mann–Whitney U tests were conducted to determine the significance of differences in morphometric characters between the new species and *G. jinxiuensis*. All statistical analyses were performed using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA), and differences were considered statistically significant at $P < 0.05$. Sex was determined based on male secondary sexual characters: the presence of a vocal sac and nuptial pads/spines (Fei and Ye 2016).

We also compared the morphological characters of the new taxon with other species of *Gracixalus*. Comparative data were obtained from the literature for 20 species of *Gracixalus* (Table 2, Suppl. material 1).

Results

Phylogenetic analyses

The obtained alignment of 16S sequences was 431 bp in length after cutting off both ragged sides. Both BI and ML analyses recovered this lineage as the sister to *G. jinxiuensis* (Fig. 2). The uncorrected *p*-distance between the unnamed taxon and its most closely related congeners was 3.4% (the new taxon vs. *G. jinxiuensis*), which was higher than between many pairs of congeners; for example, 0.7% between *G. carinensis* and *G. sapaensis*, 2.4% between *G. tianlinensis* and *G. ziegleri*, and 2.4% between *G. ziegleri* and *G. sapaensis* (Suppl. material 2).

Therefore, molecular phylogenetic analyses indicated that the population of *Gracixalus* from Leishan County, Guizhou Province, China, was distinct from its congeners.

Morphological analyses

The results of the U-test indicated that in males, the undescribed taxon was significantly different from *G. jinxiuensis* in many morphometric characters (all P values < 0.05; Table 3). In PCA for males, the total variation of the first two principal components was 72.2%. In the two-dimensional plots of PC1 vs. PC2, the undescribed taxon could be separated from *G. jinxiuensis* (Fig. 3). Detailed morphological comparisons revealed discrete diagnostic characters between the undescribed taxon and its congeners.

Therefore, adopt integrative taxonomy approaches with evidence from molecular and morphological data to take the decision to describe the unidentified taxon as a new species described herein.

Taxonomic accounts

Gracixalus weii sp. nov.

https://zoobank.org/482B7074-5F70-40B5-8C55-32AE1490BA04

Holotype. • MT LS20240518008 adult male, collected by Jing Liu on 18 May 2024 in Leishan County (26.3733°N,

Table 2. References for morphological characters for congeners of the genus Gracixalus.

ID	*Gracixalus* species	Citation
1	*G. ananjevae* (Matsui & Orlov, 2004)	Matsui and Orlov 2004
2	*G. carinensis* (Boulenger, 1893)	Boulenger, 1893
3	*G. gracilipes* (Bourret, 1937)	Bourret, 1937
4	*G. guangdongensis* (Wang, Zeng, Liu & Wang, 2018)	Wang et al. 2018
5	*G. jinxiuensis* (Hu, 1978)	Hu et al. 1978
6	*G. jinggangensis* (Zeng, Zhao, Chen, Chen, Zhang & Wang, 2017)	Zeng et al. 2017
7	*G. lumarius* (Rowley, Le, Dau, Hoang & Cao, 2014)	Rowley et al. 2014
8	*G. medogensis* (Ye & Hu, 1984)	Ye and Hu1984
9	*G. nonggangensis* (Mo, Zhang, Luo, Zhou & Chen, 2013)	Mo et al. 2013
10	*G. patkaiensis* (Boruah, Deepak, Patel, Jithin, Yomcha & Das, 2023)	Boruah et al. 2023
11	*G. quangi* (Rowley, Dau, Nguyen, Cao & Nguyen, 2011)	Rowley et al. 2011
12	*G. quyeti* (Nguyen, Hendrix, Böhme, Vu & Ziegler, 2008)	Nguyen et al. 2008
13	*G. sapaensis* (Matsui, Ohler, Eto & Nguyen, 2017)	Matsui et al. 2017
14	*G. seesom* (Matsui, Khonsue, Panha & Eto, 2015)	Matsui et al. 2015
15	*G. supercornutus* (Orlov, Ho & Nguyen, 2004)	Orlov et al. 2004
16	*G. tianlinensis* (Chen, Bei, Liao, Zhou & Mo, 2018)	Chen et al. 2018
17	*G. trieng* (Rowley, Le, Hoang, Cao & Dau, 2020)	Rowley et al. 2020
18	*G. truongi* (Tran, Pham, Le, Nguyen, Ziegler & Pham, 2023)	Tran et al. 2023
19	*G. yunnanensis* (Yu, Li, Wang, Rao, Wu & Yang, 2019)	Yu et al. 2019
20	*G. ziegleri* (Le, Do, Tran, Nguyen, Orlov, Ninh & Nguyen, 2021)	Le et al. 2021

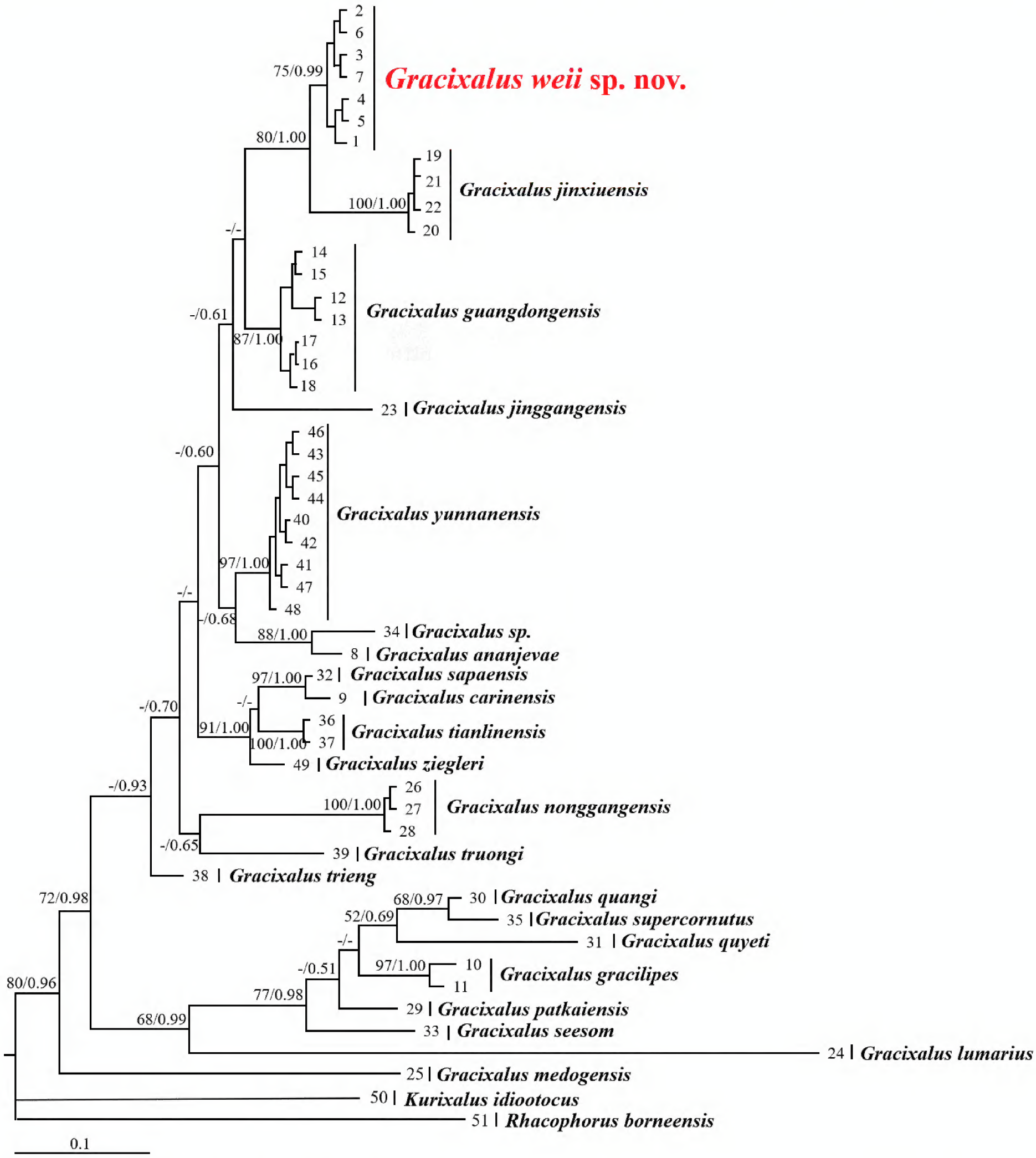

Figure 2. Maximum likelihood (ML) tree of *Gracixalus* based on the 16S gene. ML bootstrap supports (BS)/Bayesian posterior probability (BPP) were denoted beside each node, and "-" denotes BS < 50% or BPP < 0.60. Samples 1–51 refer to Table 1.

108.1967°E; elevation 1570 m a.s.l.), Guizhou Province, China.

Paratypes. • One male MT LS20230805003 was collected by Chaobo Feng on 5 August 2023 • two females, MT LS20240518009 and MT LS20240518010, were collected by Jing Liu on 18 May 2024 • and three males, MT LS20240517001, MT LS20240517002, and MT LS20240517003, were collected by Caichun Peng on 18 May 2024. All of the paratypes were collected from the same place as the holotype.

Description of holotype. Adult male (Figs 4, 5) of moderate body size, SVL 31.4 mm. The head width was larger than the head length (HDW: HDL = 1.08); the triangular snout showed a pointed appearance in the dorsal view, rounded in the profile, and projecting beyond the margin of the lower jaw; the canthus rostralis was distinct and rounded; the loreal region was oblique and concave; nostrils were oval and significantly protuberant and were closer to the tip of the snout than to the eye; the eyes were large with horizontal pupils; the eye diameter was

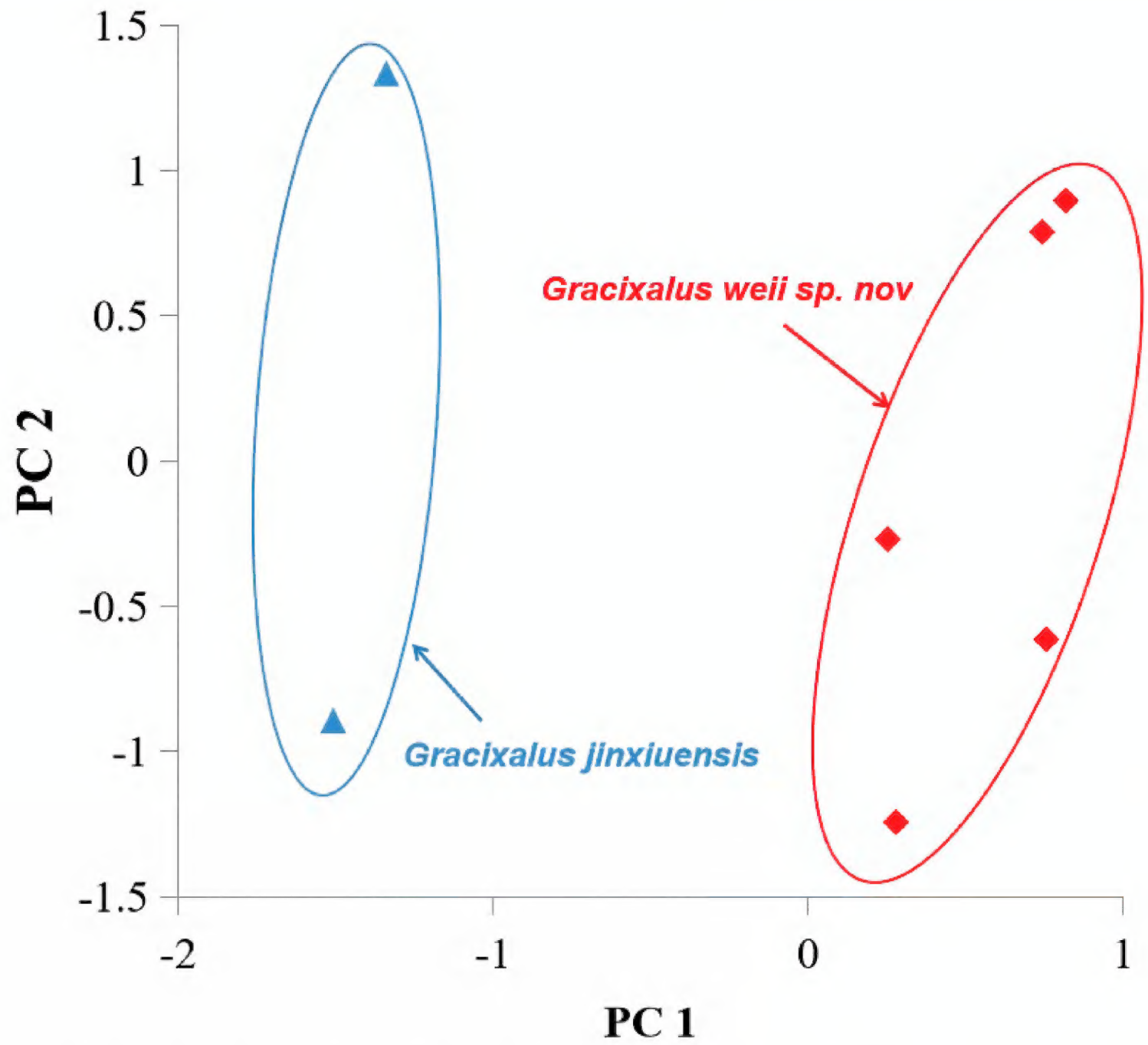

Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for males of *Gracixalus weii* sp. nov. and *Gracixalus jinxiuensis* from a principal component analysis.

Table 3. Measurements of the adult specimens of *Gracixalus weii* sp. nov. Comparison of male *Gracixalus weii* sp. nov. (PW) and *Gracixalus jinxiuensis* (PJ). Units are given in mm. *P*-values less than 0.05 indicate significant differences. See abbreviations for the morphological characters in the Materials and methods section.

Measurements	*Gracixalus weii* sp. nov.			*Gracixalus jinxiuensis*	*P*-value from Mann-Whitney U test PW vs. PJ
	male (N = 5)		female (N = 2)	male (N = 2)	
	Range	Mean ± SD	Range	Range	
SVL	30.1–34.0	31.9 ± 1.4	35.2–36.0	27.4–30.3	0.065
HDL	9.5–10.8	10.3 ± 0.5	11.3–11.8	9.9–10.3	0.026
HDW	11.6–12.8	12.1 ± 0.4	12.4–13.1	10.7–10.9	0.684
SL	4.7–5.1	4.9 ± 0.2	5.2–5.4	4.9–5.0	0.008
IND	3.8–4.5	4.1 ± 0.3	4.0–4.8	2.6–3.1	0.001
IOD	3.4–4.2	3.7 ± 0.4	3.7–4.4	3.6–4.3	0.037
UEW	2.3–2.9	2.5 ± 0.2	2.7–3.0	2.6–2.7	0.103
ED	3.3–3.7	3.6 ± 0.1	4.4–4.9	3.9–4.0	0.007
TYD	2.2–2.6	2.4 ± 0.2	2.7–3.4	1.6–1.8	0.002
LAL	15.9–17.6	16.7 ± 0.7	18.0–18.2	13.4–14.7	0.054
LW	2.5–3.0	2.7 ± 0.2	2.7–2.8	2.3–2.4	0.460
ML	9.6–10.6	10.2 ± 0.4	10.9–11.3	8.2–9.1	0.148
HLL	45.6–51.1	48.6 ± 2.3	50.0–50.3	40.2–44.7	0.128
THL	13.4–15.2	14.2 ± 0.8	15.0–15.5	12.6–12.6	0.663
TL	14.9–17.2	15.8 ± 0.9	17.3–17.4	13.8–13.8	0.381
TW	3.1–4.1	3.7 ± 0.4	4.0–4.2	2.6–3.1	0.110
TFL	21.9–23.7	22.6 ± 0.8	24.5–24.6	18.2–18.6	0.010
FL	12.1–14.8	13.6 ± 1.0	14.7–15.2	11.5–12.2	0.332

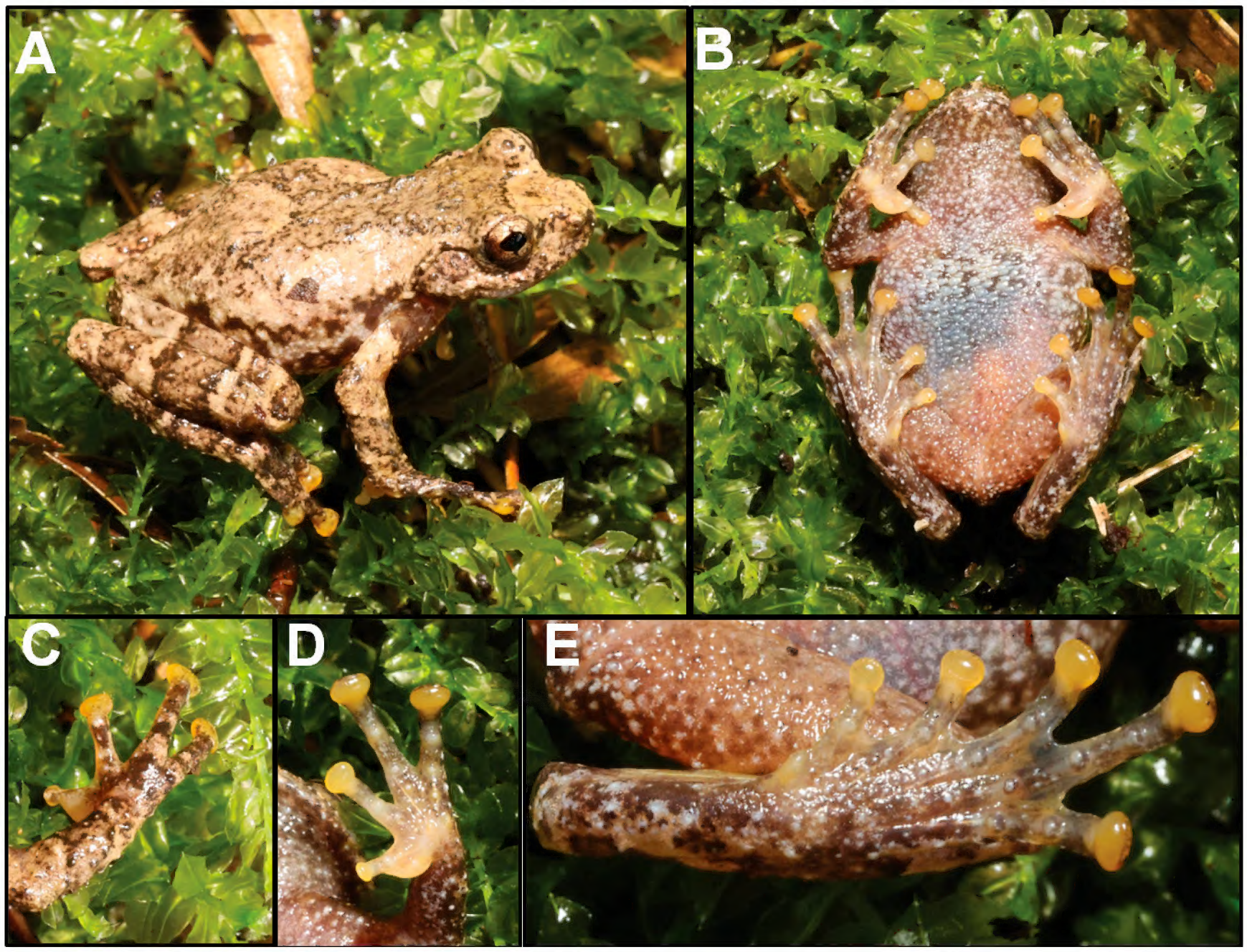

Figure 4. Photos of the holotype MT LS20240518008 of *Gracixalus weii* sp. nov. in life. **A.** Dorsal view; **B.** Ventral view; **C.** Dorsal view of hand; **D.** Ventral view of hand; **E.** Ventral view of foot. (Photo **A–E** by Shize Li).

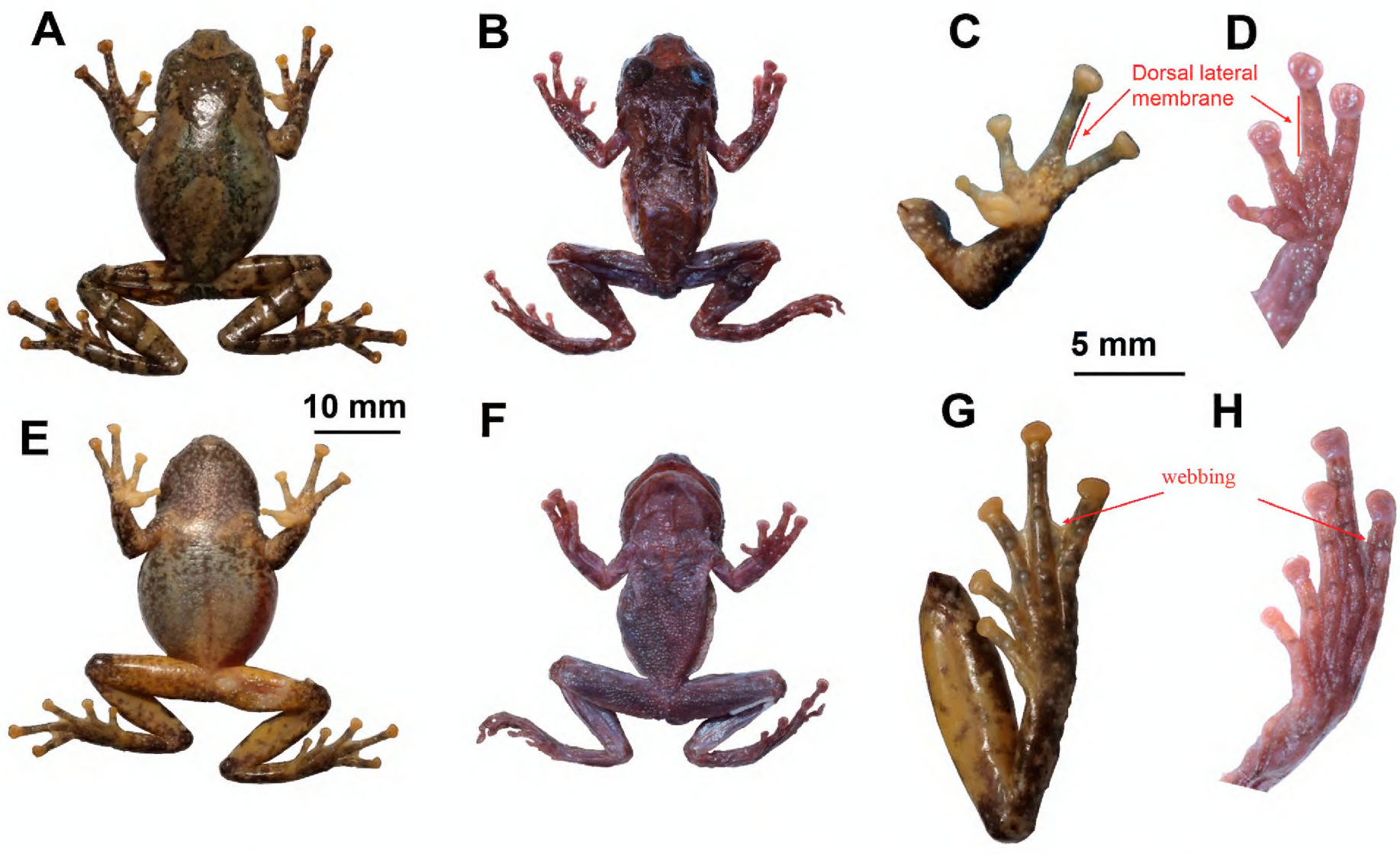

Figure 5. Comparison of morphological characteristics between the holotype specimen of *Gracixalus weii* sp. nov. (MT LS20240518008; **A, C, E, G**) and *Gracixalus jinxiuensis* (CIB58795(660386); **B, D, F, H**). **A, B.** Dorsal view; **C, D.** Ventral view of hand; **E, F.** Ventral view; **G, H.** Ventral view of foot. (Photo **A–H** by Jing Liu).

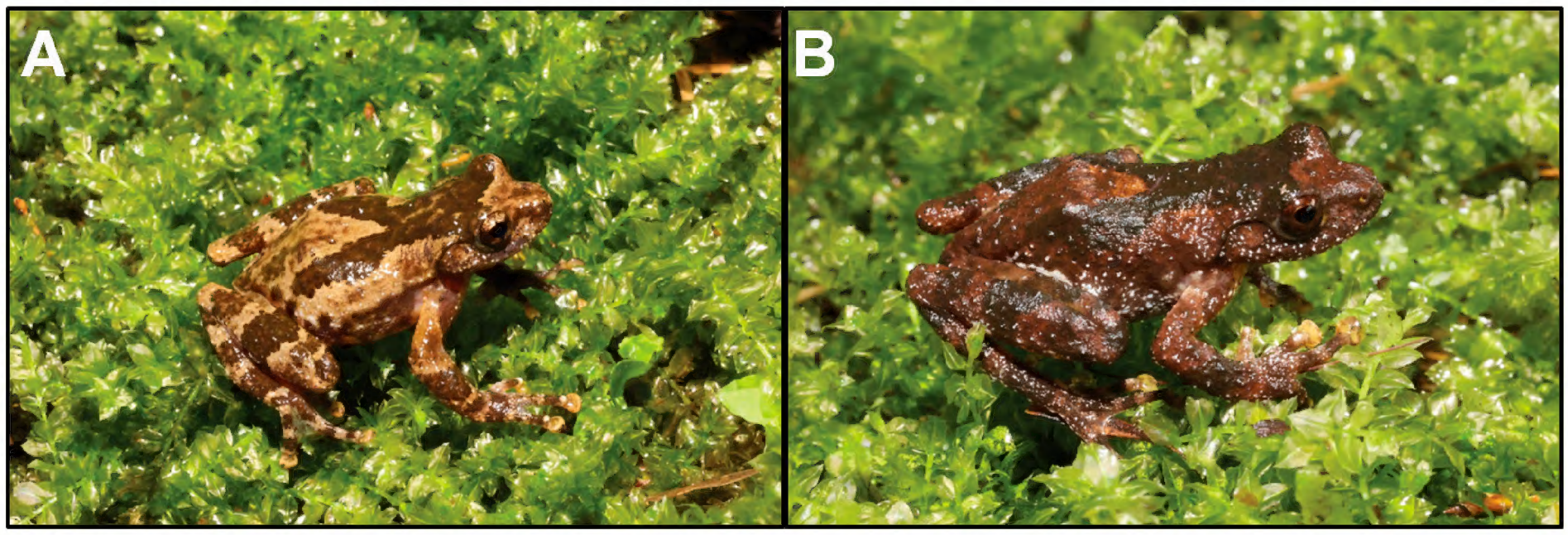

Figure 6. Color variation in *Gracixalus weii* sp. nov. **A.** Dorsolateral view of the male specimen MT LS20240517002; **B.** Dorsolateral view of the male specimen MT LS20240518010. (Photo **A, B** by Shize Li).

0.68 times that of snout length; the distinct tympanum was 0.69 times the eye diameter; the supratympanic fold was distinct, extending from the posterior corner of eye to above the insertion of the arm; no vomerine teeth were observed; the tongue was deeply notched behind.

The forelimbs were robust (LW/SVL = 0.08); the lower arm and hand beyond one-second of body length (LAL/SVL = 0.51); fingers were dorsoventrally compressed, with webbing rudimentary but with narrow lateral fringes on the outer edge of all fingers, relative finger lengths were ordered as I < II < IV < III; the tips of all fingers showed well-developed disks with horizontal circummarginal grooves; markedly elevated, prominent, and rounded subarticular tubercles were found on the fingers (one on fingers I and II, and two on fingers III and IV); supernumerary tubercles were present; the prepollex was distinct, oval; a nuptial pad was observed on the dorsolateral surface of the first finger.

Hindlimbs were 1.5 times the SVL; the tibiotarsal articulation reached the mid-eye level when legs were stretched forward; the tibias were longer than the thigh length; heels overlapped when thighs were positioned at right angles to the body; toes were slender, relative lengths I < II < III < V < IV; tips of toes showed expanded discs with distinct transverse circummarginal grooves. Distinct, rounded subarticular tubercles were seen on the fingers (one on fingers I and II, two on fingers III and V, three on finger IV); webbing formula I (2), (2½) II (1⅔), (3) III (2), (3) IV (3), (2) V; the sole was smooth with small tubercles; the inner metatarsal tubercle was elongated, ellipsoid; the outer metatarsal tubercle and the inner tarsal fold were absent.

The skin on the dorsal surface of the head, dorsum, and dorsal surfaces of the limbs was rough and sparsely scattered with conical tubercles; the ventral surface of the bell and limbs was rough with small tubercles.

Coloration in life. In life, the dorsal surface ranged from brown to beige, with an inversed Y-shaped dark brown marking from the interorbital region, bifurcating into two branches on the shoulder and extending posteriorly; the dorsal surface of the arm and limbs showed dark brown transverse bands; several large dark blotches were observed on the ventrolateral region of flanks; the ventral surface of the throat, chest, and forelimbs was flesh-colored with small dark specks; the belly had large white tubercles; the ventral surface of the hindlimbs was brown; the disks at the tips of the fingers and toes were a pale yellow.

Color in preservative. The dorsal surface of the head, body, and limbs was brown; the Y-shaped marking and transverse bands were black; the ventral surface of the chest and belly faded to white, with a gray throat (Fig. 5).

Variation. The measurements of all specimens are listed in Table 3 and Suppl. material 3. All specimens were very similar in morphology. However, the dorsum of MT LS20240517002 was brown, with a dark brown inversed Y-shaped marking on the dorsum (Fig. 6A); in MT LS20240518010, the dorsum and dorsal surfaces of the limbs were chocolate (Fig. 6B).

Secondary sexual characters. Adult females were found to be larger than adult males. The adult males showed a single subgular vocal sac. In breeding, the nuptial pad was present on the dorsal surface of the bases of the first finger.

Diagnosis. *Gracixalus weii* sp. nov. was assigned to the genus *Gracixalus* based on molecular data and morphological characteristics. Morphologically, the new species presented an intercalary cartilage between the terminal and penultimate phalanges of digits. The tips of the digits expanded into large disks with circummarginal grooves, the vomerine teeth were absent, and the pupil was horizontal (Delorme et al. 2005).

Gracixalus weii sp. nov. can be distinguished from other congeners by the following characteristics: (1) moderate body size (SVL 30.1–34.0 mm in males and 35.2–36.0 mm in females); (2) head width larger than head length; (3) distinct tympanum; (4) brown to beige dorsum, with an inversed Y-shaped dark brown marking; (5) rough skin of the dorsal and lateral surfaces of the head, body, and limbs, sparsely scattered with tubercles; (6) toes with moderately developed webbing, while finger webbing was rudimentary; (7) nuptial pads on base

of finger I; (8) males had a single subgular vocal sac; (9) tibiotarsal articulation reaching the mid-eye when leg stretched forward; (10) heels overlapping when legs at right angle to body.

Morphological comparisons. A summary of morphological comparisons is present in Suppl. material 1. *Gracixalus weii* sp. nov. appears to be most closely related to *G. jinxiuensis* but differs from it by having a relatively larger size, SVL 30.1–34.0 mm in adult males and 35.2–36.0 mm in adult females (vs. 24.2–26.3 mm in males, 28.0–29.2 mm in females); head width larger than head length (vs. the length and width of the head are almost equal); sole of foot and palmar smooth with sparse small tubercles (vs. rough with dense large tubercles); heels overlapped when thighs are positioned at right angles to the body (vs. heels just meeting); tibiotarsal articulation reaching the level of mid-eye when leg stretched forward (vs. posterior angle of eye); *Gracixalus weii* sp. nov. differs from *G. jinxiuensis* by having significantly lower ratios of ED, IOD, and SL to SVL in males and having significantly higher ratios of HDL, IND, TYD, and TFL to SVL in males (all p-values < 0.05; Table 3).

Gracixalus weii sp. nov. can be distinguished from *G. ananjevae* by having a smaller body size in females SVL 35.2–36.0 mm in adult females (vs. 43.4 mm), with distinctive conical tubercles on dorsum (vs. absent) and skin of throat granular (vs. plain) ; from *G. carinensis* by having distinctive conical tubercles on dorsum (vs. absent) ; from *G. gracilipes* by having bigger body size both in males and females (SVL 30.1–34.0 mm in males and 35.2–36.0 mm in females vs. 20.0–24.0 mm in males and 26.4–28.8 mm in females), distinctive conical tubercles present on dorsum (versus absent), dorsal surface brown (vs. greenish), tibiotarsal articulation reaching to mid-eye (vs. reaching to between eye and nostril) and throat granular (vs. smooth); from *G. guangdongensis* by having distinctive conical tubercles on dorsum (vs. absent), finger webbing rudimentary (vs. absent) and linea masculine absent (vs. present); from *G. jinggangensis* by having distinctive conical tubercles on dorsum (vs. absent) and nuptial pads present only on finger I (vs. nuptial pads present on fingers I and II); from *G. lumarius* by having a smaller body size in males (SVL 30.1–34.0 mm vs. 38.9–41.6 mm) and males with an internal vocal sac (vs. an external vocal sac); from *G. medogensis* by having a bigger body size in males (SVL 30.1–34.0 mm vs. 26.5 mm), having distinctive conical tubercles on dorsum (vs. absent) and linea masculine absent (vs. present); from *G. nonggangensis* by having a bigger body size in females (SVL 35.2–36.0 mm vs. 26.8–27.3 mm), having distinctive conical tubercles on dorsum (vs. absent), finger webbing rudimentary (vs. absent), nuptial pads present on finger I (vs. nuptial pads absent) and tibiotarsal articulation reaching to mid-eye (vs. reaching to tip of snout); from *G. patkaiensis* by having bigger body size in males (SVL 30.1–34.0 mm vs. 23.6–26.5 mm), finger webbing rudimentary (vs. absent), males with an internal vocal sac (vs. an external vocal sac) and tibiotarsal articulation reaching to mid-eye (vs. reaching to tip of snout) ; from *G. quangi* by having bigger body size both in males and females (SVL 30.1–34.0 mm in adult males and 35.2–36.0 mm in adult females vs. 21.0–24.0 mm in males and 26.8–27.3 mm in females), finger webbing rudimentary (vs. absent), males with an internal vocal sac (vs. an external vocal sac) and skin of throat granular (vs. absent); from *G. quangi* by having bigger body size in males (SVL 30.1–34.0 mm vs. 28.5 mm), skin of throat granular (vs. absent) and tibiotarsal articulation reaching to mid-eye (vs. reaching to snout); from *G. sapaensis* by having bigger body size in males (SVL 30.1–34.0 mm vs. 20.8–29.6 mm) and with distinctive conical tubercles on dorsum (vs. absent) ; from *G. seesom* by having bigger body size both in males and females (SVL 30.1–34.0 mm in males and 35.2–36.0 mm in females vs. 21.6–23.0 mm in males and 23.2–25.4 mm in females), with distinctive conical tubercles on dorsum (vs. absent), males with an internal vocal sac (vs. an external vocal sac) and tibiotarsal articulation reaching to mid-eye (vs. reaching between eye and nostril); from *G. supercornutus* by having bigger body size in males (SVL 30.1–34.0 mm vs. 22.0–24.1 mm); from *G. tianlinensis* by having distinctive conical tubercles on dorsum (vs. absent), males with an internal vocal sac (vs. an external vocal sac) and nuptial pads present only on finger I (vs. nuptial pads present on fingers I and II); from *G. trieng* by having smaller body size in males (SVL 30.1–34.0 mm vs. 37.2–41.4 mm), finger webbing rudimentary (vs. absent) and males with an internal vocal sac (vs. an external vocal sac) ; from *G. truongi* by having smaller body size in females (SVL 35.2–36.0 mm vs. 37.6–39.3 mm), finger webbing rudimentary (vs. absent), having distinctive conical tubercles on dorsum (vs. absent) and tibiotarsal articulation reaching to mid-eye (vs. reaching between eye and nostril); from *G. yunnanensis* by males with an internal vocal sac (vs. an external vocal sac); freom *G. ziegleri* by tibiotarsal articulation reaching to mid-eye (vs. reaching tip of snout).

Distribution and habitats. At present, *Gracixalus weii* sp. nov. is only found in Leigongshan Nature Reserve, Leishan County, Guizhou Province, China. The population inhabits the mountain forests at elevations between 1600 and 1800 m and is often found on bamboo near streams (Fig. 7). *Boulenophrys leishanensis* Li, Xu, Liu, Jiang, Wei & Wang, 2018; *B. spinata* Liu & Hu, 1973; *Leptobrachella wulingensis* Qian, Xia, Cao, Xiao & Yang, 2020; and *Leptobrachium leishanensis* Liu & Hu, 1973 were also found in the type locality of the new species. After investigation, we only heard the calls of Gracixalus in mid to late May, and no calls were heard in other seasons, so we speculate that the breeding season may be in mid to late May.

Etymology. The specific epithet "*weii*" was given in honor of Professor Gang Wei from Guiyang University, in recognition of his contributions to the systematic studies and biodiversity conservation of amphibians in China.

Figure 7. Habitat of *Gracixalus weii* sp. nov. in the type locality, Leishan County, Guizhou Province, China. (Photo by Jing Liu).

Discussion

Over the last decade, morphological and phylogenetic analyses have facilitated the identification of species in the genus *Gracixalus*, which has increased from nine to twenty (Boruah et al. 2023). However, recent phylogenetic analyses (Matsui et al. 2017; Chen et al. 2018) revealed that several distinct lineages in the group of *G. jinxiuensis* remain unnamed. This study described a new species from Leigongshan Nature Reserve, Guizhou Province, China, based on morphological characteristics and phylogenetic analyses. The phylogenetic tree indicated that the specimens from the Leigongshan Nature Reserve were a sister species to *G. jinxiuensis*. The genetic distance on 16S gene sequences between the new species and *G. jinxiuensis* was 3.4% (Suppl. material 2), which was higher than the threshold of interspecific divergence (3%) on 16S genes proposed by Fouquet et al. (2007) and Vieites et al. (2009) for amphibian candidate species. This level of 3.4% genetic divergence was also much higher than that between many other sister species in *Gracixalus* (Suppl. material 2). Furthermore, our detailed morphological comparisons revealed a series of characteristics that could be used to differentiate the species from its congeners. Collectively, the integrative evidence consistently supports the validity of the new species.

Guizhou Province is one of the richest regions in China for amphibians. In the past five years, more than 10 new species of amphibians have been discovered in Guizhou (Amphibia China 2024). However, the *Gracixalus* species in Guizhou has been discovered for the first time to date, indicating that the diversity of amphibians in Guizhou is still underestimated. In recent years, our research group has conducted multiple surveys on the amphibians of Leigongshan Nature Reserve and has identified three new species (Li et al. 2018, 2019, 2024). This time, our team has discovered *Gracixalus weii* sp. nov. in Leigong Mountain. This implies that this species may be found in the areas surrounding Leigong Mountain. In addition, more attention should be paid to those species with good concealment, which are hard to find.

Acknowledgements

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Supplementary material 1

Morphological characters for comparisons among *Gracixalus* species

Authors: Jing Liu, Cai-Chun Peng, Bin Wang, Chao-Bo Feng, Tuo Shen, Shi-Ze Li, Ji-Jun Chen, Hai-Jun Su, Xiu-Jun Tang

Data type: xls

Explanation note: "?" = not known or not clearly defined.

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Link: https://doi.org/10.3897/zse.101.133735.suppl1

Supplementary material 2

Uncorrected *p*-distances between species in the *Gracixalus* based on the 16S gene sequences

Authors: Jing Liu, Cai-Chun Peng, Bin Wang, Chao-Bo Feng, Tuo Shen, Shi-Ze Li, Ji-Jun Chen, Hai-Jun Su, Xiu-Jun Tang

Data type: xlsx

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Supplementary material 3

Individual measurements of the adult specimens of *Gracixalus weii* sp. nov.

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Data type: xlsx

Explanation note: Units are given in mm. See abbreviations for the morphological characters in the Materials and methods section.

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